LIPOSOMES FOR PROTECTION AGAINST
TOXIC COMPOUNDS

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001]   This application claims the benefit of U.S. Provisional Patent Application No. 60/425,814, filed November 13, 2003.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002]   The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of CA 77495 awarded by the National Institutes of Health.

BACKGROUND OF THE INVENTION

[0003]   The present invention relates to the bioremediation (e.g., removal) of toxic compounds, and more specifically to the protection of mammals and the environment against toxic organic compounds, their related species and metabolites, especially those that result from damage or stress.

[0004]   Toxic compounds can harm both humans and the environment. Toxic compounds are often referred to as xenobiotics. These compounds are generally highly toxic to life forms (including humans), are exceedingly difficult to dispose of, and are of major concern to industry (because of the cost and/or difficulty of treatment) and to regulatory agencies. Toxic compounds may be by-products of larger molecules, or may result from damage to biological molecules (e.g., stress that is drug-induced, chemically-induced, or physiologically induced). The damage may also be physiologic in nature (e.g., the result of an oxidative or alkylating nature) or be produced by radiation.

[0005]   In the environment, a large source of xenobiotics arises from the manufacturing of chemicals (e.g., benzene, toluene, styrene, pesticides, dioxins, halogenated organic compounds such as pentachlorophenol and PCB, and polybrominated diphenyl ethers). Toxic environmental pollutants are often present in

process waste streams, and may be present in larger quantities after spills, or in the soil and water associated with abandoned or poorly controlled industrial sites.

[0006]   Environmental toxic compounds, whether in process waste streams or in spills, are now generally treated by physical, chemical or biological means. One means includes trying to physically remove the toxic materials, e.g., from air and water streams, by contacting the toxins with activated carbon particles contained within adsorption columns. A significant drawback of this approach is that the xenobiotics adsorbed onto the carbon are not destroyed, only physically removed from the contaminated stream, and therefore some subsequent disposal method to destroy the toxins must still be employed. Toxic organic compounds may also be removed by chemical means (e.g., incineration); however, this approach is costly (e.g., high temperature and pressure equipment are required) and results in the release of undesirable combustion products into the atmosphere. Therefore, there remains a need to cost-effectively process environmental toxic organic compounds without adding environmental insults or wastes into the surroundings.

[0007]   Biological treatment of toxic compounds often involves the addition of the toxic material to bioreactors (i.e., tanks with aqueous microorganism suspensions) to degrade the materials to harmless end products such as carbon dioxide and water. Although potentially the lowest cost approach to xenobiotic destruction, current biological treatment of toxic organics suffers from fundamental inefficiencies. For example, the toxic material often kills the microorganisms (this is especially common with conventional wastewater treatment systems). Another drawback is that when added too slowly, microorganisms present in a biotreatment system often starve or become unable to consume the toxic compounds. Because of the above problems with current bioremediation there still remains a long-felt need to transform these toxic compounds in a more efficient, controlled, and cost-effective manner.

[0008]   In mammals, toxic compounds may arise from environmental contact, from ingestion or infusion of organic or inorganic chemicals (including pharmaceutical and herbal products), and from internal oxidative damage or stress, alkylating damage, or radiation damage. Environmental contaminants, poisons, allergy producing agents and chemicals (such as pesticide residues), toxic trace elements, certain drugs and pharmaceuticals, as well as excessive levels of other non-end product metabolites that are

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formed in biochemical reactions in the body during states of altered metabolism are examples of compounds that may produce toxic organic compounds. Mammalian syndromes, conditions, and diseases may also lead to the accumulation of these toxic compounds, examples of which include fatigue, cancer, hypotonia, depression, lassitude,

5   muscle weakness, insomnia, recurring bad dreams, intestinal complaints (myalgia), confusion, and functional nervous system problems.

[0009]   Most mammals contain instrinsic biotransformation-detoxification pathways to rid themselves of naturally occurring toxic organic compounds; however, these physiologic pathways are only efficient when biotransformation-detoxification

10   requirements are small. Under situations of stress (e.g., oxidative, alkylating, radiation) or when nonnatural chemicals are introduced, natural biotransformation-detoxification pathways are, themselves, often incapable, inefficient and ineffective at ridding the cell or the biologic system of the chemical. Often, the chemical may be initially transformed after which potentially toxic by-products then accumulate within the host and can prove

15   fatal. Attempts to protect mammals from toxic accumulation of organic compounds and their by-products are generally done after chemical insult has already occurred. The addition of chemicals, foods, vitamins, nutritional supplements or drugs may be used to try to relieve the body of the excessive toxins. Most of the additives, however, are either inefficient, costly and/or have serious deleterious side effects. For mammals, these

20   current inefficiencies and problems mean that there remains a need to aid in the protection of mammals against toxic organic compounds in an efficient, controlled, and cost-effective manner.

SUMMARY OF THE INVENTION

[0010]   The present invention solves the current problems associated with removal of

25   toxic wastes (e.g., toxic waste compounds, xenobiotics) from the environment, from biologic waste, and from mammals. The present invention identifies a novel protein that is a non-ABC transporter, referred to herein as RLIP76, that efficiently detoxifies xenobiotics by a process that catalyzes ATP. Importantly, the protein is useful in the protection of mammals against xenobiotic accumulation and for the transport of

30   xenobiotic waste in the environment often associated with industrial and chemical processing. RLIP76 is also identified as a protein involved in drug resistance and in the

protection against toxic by-products of metabolism, stress, and drugs or other organic chemicals.

[0011]   Generally, and in one form, the present invention is a method of preparing a proteoliposome comprising the step of contacting a liposome with an effective portion of

5   RLIP76 to create a proteoliposome. The liposome is generally selected at least from the group consisting of lectin, glycolipid, phospholipid, and combinations thereof. In another aspect, the proteoliposome is added to one or more toxic compounds to reduce the concentration of toxic compounds, prevent the accumulation of toxic compounds, and protect against further contamination with one or more toxic compounds. Toxic

10   compounds may be present in an organism, mammalian cell, transfected mammalian cell, bioreactor, soil, water, spill, process waste stream, manufacturing waste chemical waste, laboratory waste, hospital waste, and combinations thereof, to which the proteoliposome is then added.

[0012]   In another form, the present invention is a proteoliposomal composition

15   comprising a liposome and an effective portion of RLIP76. The proteoliposome is used to reduce the concentration of toxic compounds and may further comprise at least 4-hydroxynonenal, leukotriene, polychlorinated biphenyls, glutathione, and combinations thereof. The effective portion of RLIP76 is dependent on ATP for optimal activity. As discussed, the proteoeliposomal composition is generally used for the treatment of toxic

20   compound exposure, is capable of being transfected into a mammalian cell, and is capable of having antibodies generated against it. The composition may be applied or administered to an organism in need thereof by injection, dermal delivery, infusion, injestion, and combinations thereof and capable of producing the desired effects.

[0013]   In yet another form, the present invention is a method of reducing the effects

25   of ionizing radiation comprising the step of adding a proteoliposome to a material with ionizing radiation, wherein the proteoliposome is a liposome and an effective portion of RLIP76. Alternatively, the proteoliposome may be added before the ionizing radiation. Ionizing radiation may include x-ray radiation, gamma radiation, ultraviolet radiation, thermal radiation, nuclear radiation, and combinations thereof.

[0014]   Another form of the present invention is a kit prepared for using the proteoliposomal composition described above comprising an effective dose of a proteoliposome, wherein the proteoliposome is a liposome and an effective portion of RLIP76 and an instructional pamphlet. The kit is generally used to reduce the

5   concentration of toxic compounds and their by-products and to enhance resistance to toxic compounds.

[0015]   The benefits of RLIP76 include the environmental, chemical and biologic protection against toxic compound and xenobiotic. RLIP76 is critical in the transport of toxic compounds and xenobiotics and for enhancing resistance to drugs/chemicals and

10   their toxic by-products (e.g., chemotherapy and radiation therapy). As used herein, toxic compounds arise as by-products of chemical and manufacturing processes (e.g., waste products), metabolism, pathologic conditions, stress, radiation, and drugs/chemicals, as examples.

[0016]   Those skilled in the art will further appreciate the above-noted features and

15   advantages of the invention together with other important aspects thereof upon reading the detailed description that follows in conjunction with the drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017]   For more complete understanding of the features and advantages of the present invention, reference is now made to the detailed description of the invention along with

20   the accompanying figures, wherein:

FIGURE 1 is a schematic representation of the pathway of detoxification mechanisms of xeno- and endobiotics showing the role of a transporter such as RLIP76;

FIGURE 2 depicts human RLIP76 cDNA nucleotide sequence, deduced amino acid sequence and peptide characterization;

25   FIGURE 3 depicts the effect of heat shock and H_2O_2 exposure on GS-HNE transport in K562 cells;

FIGURE 4 depicts (A) the effect of heat shock on the H_2O_2 mediated cytotoxicity in K562 cells and (B) the protective effect of heat shock and H_2O_2 pre-treatment on H_2O_2 induced apoptosis in K562 cells;

FIGURE 5 depicts the effect of anti-RLIP76 IgG on 4-HNE mediated apoptosis in heat shock pre-conditioned cells;

FIGURE 6 depicts the effect of RLIP76 on radiation sensitivity, wherein the mean and standard deviation of values from three groups shown are: without treatment with liposomes (circle), treatment with liposomes without RLIP76 (square), and treatment with liposomes with RLIP76 (triangle); and

FIGURE 7 depicts examples of the physiological significance of RLIP76. All figures are in accordance with at least one aspect of the present invention;

FIGURE 8 depicts the knockout and genotyping strategy as embodied in one aspect of the present invention;

FIGURE 9 depicts the effect of RIP1 on radiation sensitivity in male C57 mouse as embodied in one aspect of the present invention;

FIGURE 10 depicts the effect of RIP1 knockout, radiation and gender on DOX and DNP-SG transport as embodied in one aspect of the present invention;

FIGURE 11 depicts tissue-specific effects of RIP1 knockout on parameters reflecting oxidative stress in un-irradiated animals;

FIGURE 12 depicts tissue-specific effects of RIP1 knockout on parameters reflecting oxidative stress in X-irradiated animals; and

FIGURE 13 depicts sample results of one way, two way and three way interactions of gender, genotype and radiation by ANOVA.

DETAILED DESCRIPTION OF THE INVENTION

[0018]   Although making and using various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many inventive concepts that may be embodied in a wide variety of contexts. The specific aspects and embodiments discussed herein are merely illustrative of ways to make and use the invention, and do not limit the scope of the invention.

[0019]   In the description which follows like parts may be marked throughout the specification and drawing with the same reference numerals, respectively. The drawing figures are not necessarily to scale and certain features may be shown exaggerated in scale or in somewhat generalized or schematic form in the interest of clarity and

5   conciseness.

[0020]   As used herein, a "proteoliposome" is generally a protein and lectin or glyco- or phospholipid combination that forms a spherical micellular-like or vesicular structure. The structures may form spontaneously or by chemical or mechanical manipulation, or combinations thereof. Proteoliposomes take advantage of the amphipathic nature of the

10   lipid (or lectin) that causes them to form bilayers when in solution resulting in at least one of several shapes, including: (a) spherical micelle with the tails inward, or (b) bimolecular sheets that are bilayers with hydrophobic tails sandwiched between hydrophilic head groups. In general proteoliposomes may reseal themselves when torn or broken. Proteoliposomes may contain only one lectin or lipid or a variety and combination of

15   each. Examples of phospholipids include phosphatidylcholine, sphingomyelin, phosphatidylserine, inositol phospholipids, and phosphatidylethanolamine. When used, proteoliposomes may be charged or electrically neutral and are generally used at physiological pH. They may also be structures mixed with detergent (e.g., detergent/lipid/protein, detergent/lectin/protein). Methods for preparing proteoliposomes

20   of defined lipid–protein or lectin–protein ratios and size are well-known to one of ordinary skill in the art of molecular biology and protein/lipid biochemistry.

[0021]   "Toxic compounds" as used herein may xenobiotics, radiation, toxins, waste products, by-products of larger organic or inorganic molecules and/or may result from damage to such molecules. Stress is one example of damage. Other damages may be

25   environmentally-induced, metabolically-induced, drug-induced, chemically-induced, radiation-induced, and physiologically induced, as examples. The toxic compounds may be in a mammal or occur in the environment or come from manufacturing and/or chemical processes that produce waste products. Toxic compounds, "toxic organic chemicals," and "xenobiotics" are often used interchangeably. Toxic compounds may

30   also include crude oil, crude oil fraction, an organic or inorganic chemical compound, radiation, a chemical solvent, metabolite, metabolic by-product, a chemical warfare agent, drug, drug by-product, chemical by-product, and combinations thereof.

[0022]   As used herein, an "antibody" is an immunoglobulin, a solution of identical or heterogeneous immunoglobulins, or a mixture of immunoglobulins.

[0023]   The term "protein," as used herein, is meant to include any chain of amino acids and includes peptides, polypeptides, proteins, recombinant proteins, and modified proteins, such as glycoproteins, lipoproteins, phosphoproteins, metalloproteins, and the like.

[0024]   As used herein, "an effective portion of RLIP76," is any combination of proteolytic peptide products of RLIP76 that, when combined, promotes the transport or prevents the accumulation of toxic organic compounds and/or enhances resistance to the toxic compounds. The effective portion may be a recombinant RLIP76.

[0025]   Any conventional eukaryotic or bacterial expression vectors, of which many are known in the art, may be used in the practice of this invention to transfect mammalian cells or bacterial cells with the claimed proteoliposome. "Transfection" as used herein, may refer to the incorporation of a nucleic acid or protein into a cell by any means readily known in the art of molecular biology. As examples, transfection may include incorporation by proteoliposomes, electroporation, by viral incorporation, or by a nucleic acid-containing structures (e.g., expression vector or plasmid) and combinations thereof. The eukaryotic cell expression vectors described herein may be synthesized by techniques well known to those skilled in this art. The components of the vectors such as the bacterial replicons, selection genes, enhancers, promoters, and the like may be obtained from natural sources or synthesized by known procedures. Expression vectors useful in practicing this invention may also contain inducible promoters or comprise inducible expression systems as are well known in the art. The expression vectors may be introduced into the host cells by purely conventional methods, of which several are known in the art.

[0026]   The terms "mammal" or mammalian" and "organism" are often used interchangeably throughout the discussion of the present invention.

[0027]   All technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs, unless defined otherwise. All publications mentioned herein are incorporated herein by

reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

Bioremediation

[0028]   The bioremediation or removal of toxic compounds or xenobiotics in
5   mammals is traditionally classified into two phases—Phase I and Phase II—and the detoxification process is often classified as Phase III. Phase I reactions are those catalyzed by enzymes including cytochrome P450, epoxide hydrolases, esterases, and amidases. These enzymes introduce/expose reactive groups in xenobiotics that create bioactivated metabolites that can then be conjugated to hydrophilic compounds, such as
10   glutathione (GSH), glucuronate, sulfate, etc., by Phase II enzymes. Phase II reaction products must eventually be transported to complete the detoxification process (Phase III) because accumulation of these products can cause not only toxicity but can inhibit Phase II reactions. Hence, transport mechanisms designated as Phase III are an essential component of mammalian cellular defense mechanisms against toxic chemicals or
15   xenobiotics (shown schematically in FIGURE 1).

[0029]   Both Phase I and Phase II biotransformation enzymes occur as members of multiple gene "superfamilies" that have been extensively characterized (e.g., CYP450s and glutathione S-transferases). In contrast, relatively little is known about the transporters comprising Phase III of the detoxification process. Some of the transporters
20   may belong to several superfamilies or a small family specific to eukaryotic organisms; however, these molecules are not well understood physiologically or functionally. Known transporters are ABC transporters particularly P-glycoprotein (Pgp) and the multidrug resistance associated protein (MRP1). Little is understood about any other molecules that comprise the Phase III enzymes involved in the detoxification process.

25   [0030]   The present invention has identified a non-ABC transporter, RLIP76, as a novel protein that efficiently detoxifies xenobiotics. While the protein has reported GTPase activity, the present invention discloses that RLIP76 is involved in the catalysis ATP. As presented herein, RLIP76 catalyzes ATP-dependent uphill transport of xenobiotics and their by-products. Its activity is stimulated by chemotherapeutic agents
30   and is found to have two ATP-binding sequences that, when mutated, abrogate the ATP-binding, ATPase activity and transport function of the protein. RLIP76 may be

reconstituted in proteoliposomes and mediates ATP-dependent saturable transport of xenobiotics and their by-products. Futhermore, transfection of the RLIP76 protein into mammalian cells confers resistance to chemotherapeutic agents. Cells enriched with RPLIP76 also acquire resistance to xenobiotic toxicity. In addition, RLIP76 catalyzes the

5   transport of physiologic ligands such as leukotrienes (LTC4) and the conjugate of 4-hydroxynonenal (4-HNE) and glutathione.

Transporters Of The ABC Family

[0031]   ABC transporters utilize the free energy of ATP hydrolysis to translocate substrates or allocrites across the membrane, and have Walker motifs (ATP binding sites)

10   and transmembrane domains in their sequences. Overexpression of ABC transporters has been linked with drug resistance of certain bacteria, parasites and human cancer cells. Two ABC transporter family members P-glycoprotein (Pgp or MDR1) and multidrug resistance associated protein (MRP1) are characterized with respect to this function. Overexpression of Pgp, MRP1, or both is observed in many cancer cell lines exhibiting

15   the multidrug resistance phenotype. Pgp overexpressing cancer cells exposed to a drug such as a chemotherapeutic agent (e.g., adriamycin, vinblastine, colchicines) show decreased accumulation of the drug.

[0032]   MRP, now designated as MRP1 (first characterized member of the MRP family) or ABCC1 was originally cloned from a drug resistant line selected for

20   doxorubicin (DOX) resistance. MRP1-mediated transport of the conjugates of GSH, glucuronate, and sulfate has been clearly demonstrated. MRP1 also mediates the transport of physiological GSH-conjugates (e.g., leukotrienes, GS-HNE–GSH conjugate of lipid peroxidation end product, 4-HNE). Transport of vincristine by MRP1-rich membrane vesicles has been demonstrated and this transport has been suggested to be

25   linked to GSH co-transport.

[0033]   Despite the identification of multiple families of drug transporters in the human genome, including at least 48 sequences of putative proteins having characteristics of ABC-transporters, the functional characterization of these transporters is lacking.

[0034]   The present invention describes the function of a protein, not of the ABC

30   transporter family, that has a novel role as a primary active transporter of xenobiotics,

their conjugates, toxic metabolic by-products (including drug- or physiologically induced), and other chemicals (e.g., chemotherapeutic agents), especially those involved in drug resistance. The novel protein of the present invention functions as a Ral-binding, GTPase-activating protein or RLIP76. RLIP76 function results in transport of molecules

5   associated with drug resistance and of exogenous and endogenous toxicants.

DNP-SG ATPase: A Transporter For Anionic And Cationic Xenobiotics

[0035]   DNP-SG ATPase is a protein in membranes of human cells that catalyzes ATP hydrolysis in the presence of GSH-conjugates. It was so named because S-(2,4-dinitrophenyl)glutathione (DNP-SG) stimulated its ATPase activity. The presence of

10   DNP-SG ATPase was demonstrated in all human tissues examined including liver, heart, lung, muscle, kidneys, erythrocytes, leukocytes and various human cell lines of diverse tissue origin. [*See* LaBelle EF, et al. 1988. *FEBS Lett* 228:53-6; Sharma R, et al. 1990. *Biochem Biophys Res Commun* 171:155-61; Saxena M, et al. 1992. *Arch Biochem Biophys* 298:231-7; Awasthi S, et al. 1994. *J Clin Invest* 93:958-65; Awasthi S, et al.

15   1998. *Biochemistry* 37:5231-8; Awasthi S, et al. 1998. *Biochemistry* 37:5239-48; all citations incorporated herein by reference.] DNP-SG ATPase-mediated ATP hydrolysis was stimulated not only by organic anions (e.g., DNP-SG), but by cations such as chemotherapeutic agents (e.g., doxorubicin or DOX) and their metabolites. DNP-SG ATPase catalyzed transport of anionic GSH conjugates as well as of weakly cationic

20   drugs such as DOX and colchicine (Awasthi et al. 1994, 1998a, 1998b, 1999, incorporated herein by reference).

[0036]   ATP-dependent transport of both anions and cations against a concentration gradient was demonstrated in proteoliposomes reconstituted with highly purified DNP-SG ATPase. Transport was temperature-dependent and sensitive to the osmolarity of the

25   assay medium. ATP hydrolysis was required for the transport because when ATP was replaced by its non-hydrolyzable analogue, methylene-adenosine triphosphate (Met-ATP), transport activity was abolished. This suggested that transport was directly coupled to ATP hydrolysis, and that DNP-SG ATPase was a primary active transporter. Antibodies raised against DNP-SG ATPase inhibited the transport of anions and cations

30   in inside-out vesicles (IOVs) prepared from erythrocyte membranes suggesting that the transport was specifically catalyzed by DNP-SG ATPase. On the other hand, antibodies

against MRP1or Pgp neither recognized DNP-SG ATPase in Western blots nor affected its transport activity, establishing that DNP-SG ATPase was a distinct transporter.

[0037]   A protein related to DNP-SG ATPase was also identified in rodents (Zimniak P, et al. 1992. *Arch Biochem Biophys* 292:534-8; Zimniak P, Awasthi YC. 1993. *Hepatology* 17:330-9; Pikula S, et al. 1994. *J Biol Chem* 269:27574-9; Pikula S, et al. 1994. *J Biol Chem* 269:27566-73; all citations herein incorporated by reference). Antibodies against human DNP-SG ATPase recognized the protein in rat canalicular membranes. When purified and reconstituted in proteoliposomes, it catalyzed concentrative transport of DNP-SG with kinetic parameters similar to those of human DNP-SG ATPase. The biochemical characteristics of the rat transporter and human DNP-SG ATPase were clearly distinct from the MRP2 from human and rats. These results clearly demonstrate that in mammals, other transporter(s) besides MRP2 is/are present.

Cloning Of DNP-SG ATPase And Its Identity With RLIP76

[0038]   The molecular identity of DNP-SG ATPase remained elusive for over a decade because of the inherent difficulties in its purification (e.g., protein was prone to degradation, and peptides of varying chain lengths were observed in SDS gels of purified preparations, especially a 38 kDa peptide fragment). Purified preparations highly enriched in the 38 kDa peptide were found to mediate ATP-dependent, uphill transport of anions and cations in reconstituted proteoliposomes.

[0039]   Immunoscreening of a human bone marrow cDNA library using polyclonal antibodies against the 38 kDa DNP-SG ATPase peptide yielded RLIP76 (Awasthi S, et al. 2000. *Biochemistry* 39:9327-34, herein incorporated by reference). At this time RLIP was thought of as a Ral binding, GTPase-activating protein (GAP), and to bridge the Ral, Rac, Cdc42 pathways.

[0040]   The present invention now describes the expression of RLIP76 in *E. coli* that shows the recombinant protein readily undergoes degradation, yielding peptide fragments in SDS gel dependent on the conditions of purification, including a 95 kDa band and 38 kDa fragment. All the fragments are recognized by antibodies raised against DNP-SG ATPase and have internal sequences of RLIP76 (FIGURE 2), demonstrating that these

fragments originate from RLIP76 and result from proteolytic processing. Primary
fragments are the C-RLIP76$^{410-654}$ and N-RLIP$^{1-367}$ derived from the C- and N-terminus of
RLIP76, respectively (Awasthi S, et al. 2001. *Biochemistry* **40**:4159-68, herein
incorporated by reference).

5   [0041]   For FIGURE 2, human bone marrow cDNA lambda gt11 expression library
was screened with antibody against human DNP-SG ATPase, the positive plaques were
purified and the recombinant Lambda DNA were sequenced and sequence comparisons
with published sequences were generated by the Blast Program available as a network
service from the National Center for Biotechnology Information, NIH, such that the
10   results showed the DNA sequence from the positive plaque was the same as the human
RLIP76 protein mRNA coding sequence. The encoding sequence of RLIP76 was
subcloned into prokaryotic expression vector pET30 and the recombinant RLIP76 was
purified and sequenced and the deduced amino acid sequence was analyzed with the help
of the Wisconsin Genetics Computer Group with different sequence identifications that
15   include experimentally determined sequences of RLIP76 peptides obtained during
purification (e.g., Leucine zipper pattern, N-myristoylation site, Trypsin cut site,
Chymotrypsin site, Protein kinase C phosphorylation site, Tyrosine kinase
phosphorylation site, N-Glycosylation site; cAMP-dependent protein kinase site, cGMP-
dependent protein kinase site, and Casein kinase II phosphorylation site).

20   RLIP76 Mediates ATP-Dependent Transport Of Organic Anions And Cations

[0042]   DNP-SG ATPase and RLIP76 may be, in many species, the same protein.
Hence, recombinant RLIP76 (rec-RLIP76) shows constitutive ATPase activity stimulated
by anionic (e.g., DNP-SG) and cationic (e.g., DOX) ligands with similar K_m. Purified
rec-RLIP76 reconstituted in proteoliposome (e.g., with asolectin or phospholipids of
25   defined composition) catalyzes ATP dependent, uphill transport of anionic conjugates
(e.g., DNP-SG, GS-HNE) and cationic amphiphilic drugs (e.g., DOX and daunomycin)
such as those used in cancer chemotherapy. The results show that the mechanism through
which RLIP76 transports charged chemicals (e.g., anthracyclines, vincristine) is distinct
from that of MRP1. RLIP76 is not selective, it transposes both anions as well as cations.
30   More importantly, the transport does not require GSH co-transport.

[0043]   TABLE 1 summarizes structural characteristics, chromosomal location, tissue localization and substrate profiles of RLIP76, MRP1 and Pgp. The TABLE shows that RLIP76 does not share structural attributes with MRP1 or Pgp.

TABLE 1. Comparison of the characteristics of RLIP76 with Pgp (MDR1) and MRP1

	RLIP76	MDR1 (Pgp)	MRP1
Mol. Weight	76kDa	170kDa	190kDa
Chromosomal Location	Chromosome 18	Chromosome 7	Chromosome 16
Topology	No clearly defined TMDs. One NBD each in the N and C-terminal domains are distinct from Walker A and B motifs.	2 TMDs and 2 NBDs with Walker A and B motifs.	2 TMDs similar to Pgp with an extra TMD0 connected with L0 loop. 2 NBDs with Walker A and B motifs.
Expression in human tissues	Ubiquitously expressed in mammalian tissue: erythrocytes, liver, lung, bone, muscle, kidney, and from cultured cells of mammalian origin.	Widely expressed in human tissue: liver, kidney, brain, pancreas, colon adrenal gland, small intestine.	Widely expressed in human tissue: epithelia, muscle cells and macrophages.
Localization in human tissues	Plasma membrane, nuclear membrane and cytoplasm.	Apical surface of epithelia (normal tissue); plasma membrane (malignant cells).	Cytoplasmic or unidentified vesicular fraction (normal); plasma membrane (malignant cells).
Transport allocrites (example of substrates)	Cations and anions; GSH-conjugates, glucuronides, vinca-alkaloids, anthracyclins; GSH not required for co-transport.	Vinca-alkaloids, anthracyclins, taxanes GSH not required for co-transport.	GSH-conjugates, glucuronides, bile salts; GSH co-transport required for vinca-alkaloids, anthracyclins

5   Abbreviations: TMD = trans membrane domain; NBD = nucleotide binding domain.

[0044]   As described herein, physiologic significance of the ATP-dependent transport of both anions and cations by RLIP76 was confirmed by transfection experiments. Cells overexpressing RLIP76 show increased efflux of anions and cations (e.g., DOX, GS-

10   HNE, leukotrienes) and acquired resistance to both DOX and 4-HNE induced cytotoxicity.

[0045]   The transport of DOX is demonstrated in crude erythrocyte membrane

vesicles. Addition of purified protein to crude erythrocyte membrane vesicles resulted in

increased ATP-dependent DOX-transport in these vesicles in a manner linearly dependent

on the amount of purified protein added. In these vesicles, DOX transport was

competitively inhibited by anionic metabolites GS-E (DNP-SG), and bilirubin-ditaurate,

as well as cationic drugs including anthracyclines (e.g., daunorubicin, mitoxantrone),

vinca alkaloids (e.g., vinblastine), and calcium channel inhibitors (e.g., verapamil). (*See*

TABLE 2)

TABLE 2. Stimulation of human erythrocyte DNP-SG ATPase (RLIP76) activities

Stimulator/Allocrite	Fold Activation	K_M (μM)
Leukotriene C4	2.7	5.3
Leukotriene D4	1.9	7.7
Leukotriene E4	2.0	10
N-acetyl Leukotriene E4	2.1	2.6
Adriamycin	2.3	2.8
Dihydroadriamycin	1.9	2
Adriamycinone	2.2	5.8
Dihydroadriamycinone	2.4	5.2
Deoxyadriamycinone	2.1	7.6
S-(methyl)-glutathione	1.4	137
S-(n-propyl) glutathione	1.5	-
S-(n-pentyl) glutathione	1.6	-
S-(n-decyl) glutathione	1.7	1528
S-(p-chlorophenacyl) glutathione	1.8	-
S-(9, 10-epoxy stearyl) glutathione	1.9	674
S-(p-nitrobenzyl) glutathione	1.9	-
S-(dinitrophenyl) glutathione	2.0	58

[0046]   ATPase activity of purified protein fractions was then measured in the absence

and presence of several stimulators. Each assay was performed with 9 replicates and ~2

μg protein was used for each determination. K_m values were obtained from double

reciprocal plots of stimulator vs. activity. For fold activations shown in TABLE 2, the

concentration of stimulator used was generally 2-fold the K_m. TABLE 2 explains the

pharmacologic and toxicologic interactions between certain cationic drugs (e.g., natural

product chemotherapy agents, calcium channel blockers, immune suppressants) and

electrophilic compounds/drugs (e.g., alkylating chemotherapy agents, endogenously

generated electrophiles from lipid oxidation) that may be metabolized to their by-products

such as GS-E. This is particularly useful because some cells (e.g., erythrocytes) do not

possess the full complement of metabolic machinery to metabolize GS-E to mercapturic acids.

Structure Of RLIP76

[0047]   Primary structure of RLIP76 reveals several interesting features. The protein
5   may be divided into four regions out of which two central domains carry a Rac1/CDC42
GAP activity and a Ral binding domain. The function of two flanking domains are still
unknown. The amino acid sequence of RLIP76 is depicted in FIGURE 2 and indicates
the presence of sites for N-glycosylation (aa 341-344), cAMP (aa113-116), cGMP-
dependent protein kinase phosphorylation (aa 650-653), tyrosine kinase phosphorylation
10   (aa 308-315), N-mysristolation (aa 21-26, aa 40-45, aa 191-196), leucine zipper pattern
(aa 547-578) and several protein kinase C phosphorylation, casein kinase II
phosphorylation, trypsin and chemotrypsine cut sites. The presence of such motifs in the
primary structure of RLIP76, and its facile proteolytic degradation shows RLIP76 to be
involved in several intra and extracellular processes (e.g., protein processing, intracellular
15   signaling, protein degradation, recognition, tagging, etc.) and that proteolytic processing
of RLIP76 is required for the multiple functions. The peptide fragments of RLIP76
individually or in association with other fragments may catalyze these various functions.
For example, N-terminal and C-terminal fragments of RLIP76, fragments that are
individually incapable of mediating ATP-dependent transport, can catalyze the transport
20   of electrically charged drugs (e.g., DOX, colchicines) when reconstituted together in
proteoliposomes.

RLIP76 Contains Two ATP-Binding Sites

[0048]   RLIP76 expressed in cultured cells or in *E. coli* undergoes facile proteolysis
during purification. Two most prominent peptides, N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$,
25   arising from the N- and C-termini of RLIP76, respectively, appear as 49kDa and 38kDa
in SDS-gels. Both these peptides display constitutive ATPase activity that may be
stimulated in the presence of the anionic or cationic ligands transported by RLIP76. Both
peptides bind ATP, as shown by photoaffinity labeling that increased in the presence of
vanadate, indicating the trapping of a reaction intermediate in the ATP binding site (data
30   not shown). None of the two fragments catalyze transport when reconstituted alone in
proteoliposomes. However, when reconstituted together, ATP-dependent transport of

charged chemicals (e.g., DNP-SG, DOX) is observed with kinetic parameters similar to those for RLIP76. The ATP binding sites in N-RLIP76$^{1-367}$ and C-RLIP76$^{410-655}$ were identified to be ^{69}GKKKGK^{74} and ^{418}GGIKDLSK^{425}, respectively. Mutations of K^{74} and K^{425} in the N-and C-terminal peptides, respectively, abrogate the ATPase activity, ATP

5   binding capacity and transport function. The sequence of these ATP binding sites are not identical to the consensus sequence for the P-loop (Walker motif).

[0049]   Unlike the ABC transporters, no transmembrane alpha-helices are evident in the RLIP76 sequence. Its association with membranes has, however, been demonstrated by immuno-histochemical studies using specific antibodies (Awasthi S, et al. 2002.

10   *Proceedings of the American Association for Cancer Research*, 43:Abst. 4717; herein incorporated by reference). The extraction of RLIP76 from cell lysates requires detergent, suggesting membrane association, a feature essential for transport.

[0050]   These findings show a greater diversity in this transporter, in terms of structural elements defining ATP binding and mode of membrane insertion, than is

15   currently accepted. In addition, the distinction between transporters for anions as opposed to neutral or cationic substrates is blunted because RLIP76 catalyzes the transport of both, and, in contrast to MRP1, does so without co-transporting GSH.

[0051]   Another intriguing aspect of RLIP76 function is that it undergoes facile proteolytic fragmentation and many of the resulting peptides may be reconstituted into an

20   active transport complex, a function that may help regulate exocytosis and membrane ruffling (data not shown).

Toxic Compounds And Xenobiotic Protection With RLIP76

[0052]   Physiologic stress or damage (e.g., mild transient heat shock or oxidative stress) induces RLIP76 activity and the activity is in advance of inducing other heat shock

25   proteins or the antioxidant enzymes, which constitute the typical stress response (Cheng JZ, et al., 2001. *J Biol Chem* 276:41213-23, incorporated herein by reference). For example, when K562 cells are exposed to a mild heat shock (about 42 degrees Centigrade for 30 minutes) or oxidative stress (about 50 μM H_2O_2 for 20 minutes) and allowed to recover for 2 hours, enhanced LPO is observed in stressed cells as compared to non-

30   stressed cells. There is a 3-fold induction of a GST isozyme, hGST5.8, that catalyzes the

conjugation of 4-HNE and GSH to GS-HNE, and a 3.7-fold induction of RLIP76 that mediates ATP-dependent transport of GS-HNE from cells. As shown in FIGURE 3, the cells preconditioned with stress transported GS-HNE at three-fold higher rate as compared to unstressed cells. This followed a greater than 3-fold induction of RLIP76 in the preconditioned cells. For FIGURE 3, K562 cells (5×10^7 cells) were exposed to 42 degrees Centigrade for 30 minutes, and allowed to recover for 2 hours in medium at 37 degrees Centigrade. Cells were pelleted and re-incubated for 10 minutes at 37 degrees Centigrade in 2 mL medium containing 20 μM [^{3}H] 4-HNE, followed by pelleting and two washes with 2 mL of phosphate-buffered saline (PBS). The supernatants and washings were discarded and the cells were incubated at 37 degrees Centigrade for 2 hours in 2 mL of 4-HNE free medium after which radioactivity was determined in the medium. The hemiacetal 3-(4-hydroxynonanyl) glutathione (inset, FIGURE 2) was isolated by HPLC and characterized by mass spectral analysis. For H_2O_2 treatment the cells were incubated for 20 minutes at 37 degrees Centigrade in media containing 50 μM H_2O_2 and after incubation, the cells were pelleted, washed free of H_2O_2, incubated in H_2O_2 free medium at 37 degrees Centigrade for 2 hours and subsequently the radioactivity was measured in the medium. For treatment with antibodies, the cells, after heat shock treatment, were allowed to recover for 1 hour and respective IgGs were added (20 μg/ml medium) and incubated at 37 degrees Centigrade for additional 1 hour, such that the cells were pelleted and [^{3}H] GS-HNE transport was measured as described above. The values in FIGURE 3 are shown as means ± S.D. (n=3 separate experiments) and * indicates statistically significant differences between treated and control cells evaluated by the Student's t test (P< 0.05).

[0053]   To confirm that RLIP76 does indeed transport the GS-HNE and not its degradation products or metabolites, the transported allocrite, hemiacetal of 3-(4-hydroxynonanyl) glutathione, was isolated from media and characterized by mass spectral analysis.

[0054]   Increased efflux of GS-HNE was blocked by coating the cells with antibodies against RLIP76, confirming that GS-HNE was transported by RLIP76. More importantly, stress pre-conditioned cells with induced hGST5.8 and RLIP76 acquired resistance to H_2O_2-mediated cytotoxicity (FIGURE 4A) and to apoptosis by (FIGURE 4B) suppressing a sustained activation of c-Jun N-terminal kinase and caspase 3. For

FIGURE 4A, aliquots (~40 µL) containing 2×10^4 control or heat shock treated cells were washed with PBS and plated into 8 replicate wells in a 96-well plate, wherein H_2O_2 (~50 uM) in 10 µL of PBS was added and the plates were incubated at 37 degrees Centigrade for 2 hours, after which ~200 µL of growth medium was added to each well.

5   Following 72 hours of incubation at 37 degrees Centigrade, the MITT assay was performed and the OD_{590} values of sample subtracted from those of respective blanks (no cells) were normalized with control values (no H_2O_2). Averages and standard deviations from three separate determinations of cytotoxicity of 4-HNE and H_2O_2 are shown in FIGURE 4A. For FIGURE 4B, 2.5×10^6 K562 cells in 5 mL medium were treated with

10   heat shock at 42 degrees Centigrade for 30 minutes, or 50 µM H_2O_2 (final concentration in medium) for 20 minutes and allowed to recover for ~2 hours in normal growth medium at 37 degrees Centigrade. The cells, pre-conditioned with heat shock or H_2O_2 treatment, were treated with heat for 2 hours and 100 µM H_2O_2 for 2 hours. DNA (~1 µg) extracted from the cells was electrophoresed on 2% agarose gels containing 10 µg/mL ethidium

15   bromide; lanes representing different treatments are marked.

[0055]   The protective effect of stress pre-conditioning against H_2O_2 or 4-HNE induced apoptosis was abrogated by coating the cells with anti-RLIP76 IgG, which inhibited the efflux of GS-HNE from cells (FIGURE. 5). For FIGURE 5, aliquots (~50-100 µL) containing $1 \sim 2 \times 10^6$ cells were fixed onto poly-L-lysine-coated slides by

20   cytospin at $500 \times g$ for 5 minutes and the TUNEL apoptosis assay was performed. Slides were analyzed by fluorescence microscope using a standard fluorescein filter and photomicrographs at 400 × magnification are presented. Apoptotic cells showed characteristic green fluorescence. FIGURE5 includes the following: Panel 1, control cells, without heat shock pre-treatment, incubated with 20 µM 4-HNE for 2 hours; Panel

25   2, control K562 cells pre-treated with heat shock (42 degrees Centigrade, 30 minutes) and allowed to recover for 2 hours at 37 degrees Centigrade; Panel 3, cell pretreated with heat shock, allowed to recover for 2 hours at 37 degrees Centigrade followed by incubation in medium containing 20 µM 4-HNE for 2 hours at 37 degrees Centigrade; Panel 4, heat shock pre-treated cells, allowed to recover for 1 hour at 37 degrees Centigrade, anti-

30   RLIP76 IgG was added to medium (20 µg/mL final concentration) and incubated for an additional 1 hour and cells were then incubated for 2 hour at 37 degrees Centigrade in medium containing 20 µM 4-HNE.

[0056]   Induction of hGST5.8 and RLIP76 by mild, transient stress and the resulting resistance of stress-pre-conditioned cell to apoptosis is a general phenomenon, because it is not limited to K562 cells, but is evident in other cells (e.g., lung cancer cells, H69, H226, human leukemia cells, HL60, human retinal pigmented epithelial cells) (data not shown). Hence, transport activity of RLIP76 regulates the intracellular levels of potential toxic by-products. Examples of toxic by-products are the lipid peroxidation products involved in apoptosis signaling, differentiation, and cell proliferation.

Radiation Protection With RLIP76

[0057]   The protective effects of RLIP76 goes beyond its protection of potentially toxic chemical substituents and their by-products. RLIP76-enriched cells are also resistant to toxicity from radiation. For example, as shown in FIGURE 6, cells enriched with RLIP76 are remarkably resistant to radiation as compared to non-enriched control cells. Here, small cell lung cancer cells (H82) were loaded with RLIP76 by incubating with RLIP76 encapsulated in artificial liposomes. They were irradiated at 500 cGy with high-energy photon (6x10 volt photon/ min) for 1.25 minutes. Cells were serially passaged daily by inoculating 0.5 x 10^7 trypan blue dye excluding cells/mL in fresh RPMI medium. For analysis, the cell density measured each day was normalized to cell density in respective non-irradiated controls.

[0058]   As such, electrophilic products of lipid peroxidase (LPO) caused by reactive oxygen species generated during radiation may partly account for cell killings by radiation. Clearly RLIP76-mediated transport of GSH-conjugates of these electrophiles provides protection from radiation. Such protection may be readily transferred to a larger scale to protect mammals against damaging radiation, including ionizing, electromagnetic, thermal, and laser, wherein either long-or short-range electrons are involved.

[0059]   Therefore, RLIP76 mediates transport of endogenously generated chemicals, metabolic products, their by-products and exogenously administered drugs or radiation, and their by products. RLIP76 mediates the transport of most chemicals and by-products that also involve GS-E (e.g. conjugate of 4-HNE). For example, RLIP76-enriched cells are resistant to toxicity in the form of chemical toxicity (organic or inorganic) or from damage (e.g., from stress, oxidation, alkylation, radiation). The function of RLIP76 via

an ATP-dependent efflux of xenobiotics (e.g., GS-E and exogenous and endogenous electrophiles) is shown in FIGURE 7. Here, xenobiotics, radiation, their metabolites, mitochondrial electron transport and metal ions generate reactive oxygen species (ROS) that can cause membrane lipid peroxidation and 4-hydroxynonenal—the toxic end

5   product of lipid peroxidation—cause DNA damage leading to mutagenesis, carcinogenesis and apoptosis as well as modulates the stress mediated signaling pathways. Clearly, RLIP76 mediates the ATP-dependent efflux of a wide variety of metabolic, stress, and pharmaceutical by-products, such as amphiphilic drugs, GSH-conjugates (GS-E) of both xeno- and endo-biotics, GS-HNE and leukotrienes, from

10   eukaryotic cells. The transport of GS-E is crucial for maintaining functionality of GSTs and glutathione reductase (GR), because these enzymes are inhibited by GS-E. RLIP76 regulates the intracellular concentrations of 4-HNE by a coordinated mechanism with cellular GSTs

RLIP76 And Multi-Drug Resistance

15   [0060]   RLIP76 is also involved in the mechanism of multidrug resistance of cancer cells. RLIP76 mediates ATP-dependent primary active transport of not only anionic compounds (e.g., GSH-conjugates), but also the cationic chemotherapeutic drugs such as DOX, daunomycin and colchicine. The protein sequence of RLIP76 is not homologous to ABC-transporters--the proteins thought to be involved in the mechanisms of multi-drug

20   resistance. RLIP76 (1) lacks any close homologs in humans; (2) displays ubiquitous expression in tissues; (3) lacks the classic nucleotide binding Walker domains; (4) has integral membrane association without clearly defined transmembrane domains; and most importantly, (5) has distinct functions not present in other transporters (e.g., has a role as a direct link to Ras/Ral/Rho and EGF-R signaling through its multifunctional nature

25   including GAP-activity and Ras/Ral/Rho-regulated effector function involved in receptor mediated endocytosis). Its multifunctional nature is likely due to the presence of multiple motifs including Rho/Rac-GAP-domain, Ral-effector domain binding motif, two distinct ATP-binding domains, H^+-ATPase domain, PKC and tyrosine kinase phosphorylation sites, and its proteolytic processing into multiple smaller peptides that participate as

30   components of macromolecular functional complexes.

[0061]   RLIP76 overexpression confers resistance to both DOX and alkylating toxins such as 4-HNE by increasing their efflux from cells. RLIP76 can also modulate stress

signaling by regulating intracellular concentrations of 4-HNE, as it is involved in stress

signaling. Antibodies against RLIP76 can block the transport of drugs and enhance

cytotoxicity of these drugs (e,g., chemotherapeutic agents) to cancer cells. The higher

resistance to DOX of non-small cell lung cancer (NSCLC) cells as compared to the small

5   cell lung cancer (SCLC) cells correlates with a higher RLIP76-mediated efflux of DOX in

NSCLC. [*See* Awasthi S, et al. 2001. In *Pharmacology and therapeutics in the new*

millenium (Gupta, S.K., ed.), pp. 713-725, Narosa Publishing House, New-Delhi, India,

incorporated herein by reference.] Coating with RLIP76 antibodies sensitizes NSCLC to

DOX by blocking their RLIP76 mediated transport. Taken together, the present invention

10   demonstrates that RLIP76 modulates drug sensitivity of cancer cells. RLIP76 is

expressed in all human tissues and cell lines examined so far, and it catalyzes the

transmembrane movement of physiologically relevant ligands as well as a wide variety of

xenobiotics irrespective of their net charge.

[0062]   The significance of RLIP76-mediated transport to the mechanisms of

15   multidrug resistance may go beyond the protection of cells through drug efflux. RLIP76

also impacts on signaling mechanisms via the modulation of the intracellular

concentration of GS-HNE and its precursor, 4-HNE, which is known to cause cell cycle

arrest and promote differentiation and apoptosis in cancer cell lines (Cheng JZ, et al.

1999. *Arch Biochem Biophys*. 372:29-36; incorporated herein by reference). In addition,

20   the effects of 4-HNE on cell cycle signaling may be concentration dependent as it can

have the opposite effect at lower concentrations where proliferation is observed in the

presence of low 4-HNE levels. The level of 4-HNE reflects the stress status of the cell,

and to convey the corresponding signal to the cell cycle and/or apoptosis machinery.

Induction of RLIP76, by damage, oxidative or chemical stress (e.g., due to anticancer

25   drugs), depletes 4-HNE and thus promotes the proliferation of cancer cells.

[0063]   RLIP76, therefore has a two-pronged effect in multi-drug resistance; in

addition to xenobiotic and other potentially toxic chemical or drug transport, RLIP76

shifts the signaling balance in favor of cell proliferation.

RLIP76 and Radiation Sensitivity Using Knockout Mice

30   [0064]   As described, RLIP76 (also referred to as RALBP1 or Ral-binding protein) is

a glutathione-conjugate transporter that is a critical component of stress-response in

cultured cells and provides protection from stressors including heat, oxidant chemicals, chemotherapeutic agents, UV irradiation and X-irradiation.

[0065]   C57B mice which carry heterozygous (+/-) or homozygous (-/-) deletion of the RIP1 gene (mouse version of RLIP76) were created. These mice were created using Cre-
5   Lox technology that can selectively suppress genes (FIGURES 8A and B). From RIP1 +/- animals, obtained from Lexicon Genetics, we established colonies of RIP1+/+, RIP1 +/-, and RIP1 -/- C57B mice by segregation and mating of animals based on genotyping by polymerase chain reaction (PCR) on tail tissue (FIGURE 8C). Western-blot analysis of mouse tissues using anti RLIP76 antibodies confirmed decreased RIP1 levels in the
10   RIP1 +/- mouse, and its absence in tissues from the RIP1 -/- mouse (FIGURE 8D).

[0066]   For FIGURE 8, the knockout and genotyping strategy is the following. The sequence around the insertion site with the up and down-stream PCR primers (in bold-underline) are shown (A). The third primer was an LTR primer (B). ~Ten weeks old C57 mice born of heterozygous x heterozygous mating were genotyped by PCR strategy, in
15   which mouse tail DNA was isolated and used as a template in PCR reaction. A sample genotyping result is given. When all three primers are used in PCR, DNA from wild-type animal should yield a 200 bp band, knockout homozygous animal should yield a 150 bp band, and knockout heterozygous animal should yield both bands. In the figure above, lane M is DNA ladder, lanes 1, 2 and 3 are from homozygous knockout, heterozygous
20   knockout and wild-type animals (C). Analysis of RLIP76 protein in tissues from wild-type and RLIP76 knockout mice by Western blot. Crude membrane fractions from several tissues were prepared and subjected to SDS-PAGE with application of 100 μg protein per lane. Gels were transblotted on to nitrocellulose membranes, followed by Western blotting using anti-RLIP76 IgG as primary antibody. The blots were developed with 4-
25   chloro-1-naphthol as chromogenic substrate. Lane 1 contained detergent extract of bacterial membranes from rec- *E.coli* expressing RLIP76 (pET-30a[+]-RLQLIP-BL21(DE3)). Lane 2 was blank. Lanes 3 -5 contained membrane extract from liver and lanes 6-8 from heart. Lanes 3 and 6 contained protein from wild-type animal, lanes 4 and 7 contained protein from heterozygous RLIP76 knockout animal, and lane 5 and 8
30   contained protein from homozygous RLIP76 knockout animals (D). β-actin expression was used as internal control.

[0067]   The present invention shows that loss of RLIP76 (shown as a RIP1 knockout) will confer sensitivity to X-irradiation, radiation sensitivity of RIP1-/- mice was compared with the RIP1+/+ by administering 500 cGy whole-body X-irradiation using a Varian Clinac Linear accelerator (2100C), followed by monitoring for survival. A

5 representative experiment (FIGURE 9A) shows a dramatic 11 day difference in median survival between RIP1-/- (0/6 surviving by day 13) as compared with RIP +/+ (2/6 surviving at day 28). These findings provide dramatic evidence for the radiation sensitivity conferred by loss of RIP1. For FIGURE 9, C57 RIP1+/+ (square) and -/- mice (diamond) were treated with 500 cGy total body X-irradiation and survival was

10 monitored. Each group had 6 animals (A). Western blot analyses of RIP1-/- mouse tissues were performed after i.p. injection of RLIP76-liposomes (B). In the upper panel, RIP1 -/-mice were treated with RLIP76-lipsomes containing 200 µg RLIP76 protein i.p. and sacrificed 48 h later. In the lower panel, RALBP1-/- mice were treated with 3 doses of 200 µg RLIP76 liposomes at time 0, 72 h, and 120 h, followed by sacrifice at 168 h.

15 Lanes labeled C are from mice treated with control liposomes without RLIP76 and R denotes mice treated with RLIP76-liposome. Tissues as indicated in the figures were homogenized and aliquots of the detergent solubilized crude membrane fraction containing 200 µg protein was subjected to SDS-PAGE, transblotted to nitrocellulose membrane using anti-RLIP76 as primary antibody and peroxidase-conjugated goat-anti-

20 rabbit IgG as secondary antibody. The blots were developed with 4-chloro-1-napthol. β-actin expression was used as loading control. RLIP76 -/- mice treated with either control liposomes (circle) or RLIP76 -liposomes (+) at day −3, day +3 and day +5 of 500 cGy total body irradiation. Survival was monitored (C).

[0068]   If loss of RIP1 was the major determining factor in this acquired radiation

25 sensitivity, replacement of this deficit should reverse radiation resistance. Therefore, a liposomal delivery system for providing recombinant human RLIP76 to the tissues of knockout animals is presented. Methods for expressing recombinant human RLIP76 in *E. coli* and purifying the expressed protein to a high purity, >96% by amino acid composition analysis, and reconstituting its transport function in artificial liposomes are

30 those commonly used by one of ordinary skill in the art. [See Awasthi et al., *Biochemistry* 39, 9327 (2000); incorporated herein by reference.] Liposomes were

prepared in sufficient quantities and administered via the intraperitoneal (i.p.) injection to RIP1-/- animals.

[0069]   A single dose of RLIP76-liposomes containing 200 μg purified RLIP76 administered i.p. followed 48 h later by sacrificing the animals and analyzing tissues immunologically for presence of RLIP76 showed convincingly that these liposomes could be used to deliver RLIP76 to all tissues of RIP1-/- mice (FIGURE 9B). Administration of 3 doses of RLIP76-liposomes at the same dose over 8 days followed by sacrifice at day 10 showed further accumulation of RLIP76 in the RIP1-/- mouse tissues (FIGURE 9C).

[0070]   These Western-blot analyses confirmed the lack of any detectable RIP1 in any tissue from the -/- mouse and presence of a band at the expected M_r of 95 kDa for intact RLIP76 in all tissues examined from mice treated with RLIP76 liposomes. The 38 kDa band represents a C-terminal proteolytic fragment of RLIP76 beginning at aa 424. Remarkably, even the brain tissue took up a significant amount of RLIP76, a finding that may have significant pharmacological implications for delivery of drugs to the brain or other organs. The RLIP76 liposomes may incorporate one or more genes and targeted markers in order to deliver the gene to the targeted organ(s) of a mammal.

[0071]   Delivery of RLIP76 to mouse tissues also results in reversal of radiation sensitivity. The example used to show this is with 12 male RIP1-/- mice randomized into two groups of 6, the first group receiving control liposomes containing no RLIP76, and the second group receiving RLIP76-liposomes administered by i.p. injection. Animals were subjected to 500-cGy whole-body X-irradiation and followed for survival. A dramatic difference is survival was observed with all 6/6 RLIP76-liposome treated animals surviving at 55 days, as compared with 0/6 control-liposome treated animals surviving by 13 days post irradiation (FIGURE 9D). Remarkably, the RIP1 -/- mice supplemented with RLIP76 had significantly improved survival as compared with even the RIP1 +/+ mice. These finding conclusively demonstrate the radiation protective effects of RLIP76.

[0072]   The mechanism for this radioprotective effect of RALBP1 was investigated in transport studies looking at the effect of RIP1 genomic deletion on GS-E transport capacity, oxidative-stress, and glutathione-linked antioxidant enzymes in animals without or with radiation. For transport studies, crude membrane inside-out vesicles (IOVs) from

different tissues were used. The reaction mixture consisted of IOVs protein, 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 4 mM $MgCl_2$ and either 4 mM ATP or an equimolar concentration of NaCl. To start the reaction, appropriate volume of radiolabeled ^{14}C-DOX or ^{3}H-DNP-SG was added. The uptake was stopped by rapid filtration of the

5   reaction mixture through 96 well nitrocellulose plate (0.45 μm pore size). After filtration, the bottoms of the nitrocellulose membranes were blotted dry with filter paper and punched out, and the associated radioactivity was measured by placing in liquid scintillation fluid. ATP-dependent uptake of either ^{14}C-DOX or ^{3}H-DNP-SG was determined by subtracting the radioactivity of the control without ATP from that of the

10   experimental containing ATP and the transport of DOX or DNP-SG was calculated in terms of pmoles/min/mg IOV protein. GSH levels and enzyme activities for GST, GPX, GR, G6PD and γGCS activities were determined in 28,000 x g supernatants of 10 % homogenate, and LOOH and TBARS were determined in whole crude homogenates using well established methods known to those of ordinary skill in the art.

15   Radioprotection

[0073]   The example used to show the radioprotective effect is a study with a 2 x 2 x 3 factorial design (radiation x gender x genotype) and three animals per group. Six groups of irradiated animals were treated with 500 cGy whole body X-irradiation, and a remaining six groups were un-irradiated. Animals were sacrificed and autopsied at day 8

20   after irradiation. Seven tissues (brain, heart, lung, liver, kidney, intestine and spleen) were examined for content of parameters of oxidative injury and glutathione-linked enzymes. GS-E and DOX transport was examined in crude membrane vesicles prepared from plasma membrane fraction of heart tissues. Data was analyzed by ANOVA with one-way, two-way and three-way interactions between the three variables (gender,

25   genotype, radiation) being compared.

[0074]   Consistent with the observed function of RLIP76 as a transporter of GS-E and DOX in cell culture studies, GS-E and DOX transport in membrane vesicles was found to be decreased in a stepwise fashion from the RIP1+/+, to RIP1+/-, to RIP1-/- mice (FIGURE 10). For FIGURE 10, DOX and DNP-SG transport was measured as

30   previously described in crude membrane vesicles from mRALBP1+/+, +/- and -/- mice heart tissues (upper two panels, where C, and R represent un-irradiated and irradiated

animals respectively, and M and F are male and female animals, respectively). Fold-changes shown in the TABLE 3 represent changes in +/- or -/- animals with respect to the +/+ animals. The values in the bold-font represent fold-change in the -/- animals as compared with the +/- animals. Blue font shows a decrease. All values presented were

5   significant at $p < 0.01$ by ANOVA.

[0075]   A greater than 80% loss of total GS-E and DOX-transport activity was seen in the RIP1-/- mice. The differences in transport rates were statistically significantly lower in the RIP1 +/- mice as compared with RIP1 +/+, and in the RIP1 -/- mice as compared with either RIP1 +/- or RIP1 +/+ mice. These findings demonstrate that RIP1 is the

10   predominant GS-E and DOX transporter in mouse tissues.

[0076]   As such, loss of RIP1 results in increased ambient levels of oxidative stress in tissues. To demonstrate, levels of two well-accepted markers of tissue oxidative stress, LOOH and TBARS, were assessed. These parameters were measured in homogenates from 7 tissues of each of 3 animals per group in all groups. The values obtained from the

15   RIP1 +/- and RIP1 -/- mouse tissues were normalized to the corresponding values from RIP1 +/+ mice to obtain fold differences. When analyzed in aggregate for all tissues (TABLE 3), significant ($p<0.01$) increase in both LOOH and TBARS was observed for both male and female animals in the RIP1 +/- animals as compared with RIP1 +/+ animals, and fold increase was greater in the RIP1 -/- as compared with the RIP1 +/+

20   animals. The increase seen in RIP1 -/- was significant when compared with either RIP1 +/+ or RIP1 +/- mice. These findings conclusively demonstrated that progressive loss of RALBP1 results in progressive increase in tissue oxidative stress..

TABLE 3. Effect of RIP1 knockout on parmaters reflecting oxidative stress

Parameter	Unirradiated				Irradiated (500 cGy)			
	+/- (Fold)		-/- (Fold)		+/- (Fold)		-/- (Fold)	
	M	F	M	F	M	F	M	F
LOOH	↑ (1.32)	↑ (1.37)	↑ (1.94) ↑ (1.47)	↑ (2.02) ↑ (1.48)	↑ (1.62)	↑ (1.63)	↑ (2.10) ↑ (1.60)	↑ (2.22) ↑ (1.63)
TBARS	↑ (1.18)	↑ (1.17)	↑ (1.68) ↑ (1.42)	↑ (1.59) ↑ (1.35)	↑ (1.43)	↑ (1.42)	↑ (1.94) ↑ (1.64)	↑ (1.83) ↑ (1.56)
GSH	↑ (1.31)	↑ (1.48)	↑ (1.45) ↑ (1.10)	↑ (1.59) ↑ (1.70)	↑ (1.46)	↑ (1.58)	↑ (1.57) ↑ (1.20)	↑ (1.76) ↑ (1.19)
GST	↓ (0.84)	↓ (0.85)	↓ (0.81) -	↓ (0.82) -	-	-	- -	- ↑ (1.11)
GPX	↓ (0.64)	↓ (0.79)	↓ (0.54) ↓ (0.85)	↓ (0.63) ↓ (0.81)	↓ (0.73)	↓ (0.88)	↓ (0.57) ↓ (0.90)	↓ (0.70) -
GR	↓ (0.82)	↓ (0.84)	↓ (0.70) ↓ (0.85)	↓ (0.77) ↓ (0.91)	-	-	↓ (0.73) ↓ (0.89)	↓ (0.76) ↓ (0.91)
G6PD	↓ (0.82)	↓ (0.88)	↓ (0.78) -	↓ (0.83) -	-	↑ (1.19)	- -	↑ (1.17) ↑ (1.33)
γ-GCS	-	-	- -	↓ (0.79) -	-	↑ (1.14)	- -	↓ (0.91) -

[0077] For TABLE 3, methods for measurement of each parameter are those used by one of ordinary skill in the art. All parameters shown were measured in triplicate in brain, heart, lung, liver, kidney, intestine and spleen from each of 3 animals per group from 12 groups (3-genotype levels x 2 gender-levels x 2 radiation levels). Radiation dose was 500 cGy administered, and animals were sacrificed on day 8. The values for fold-changes between +/+ vs. either +/- or -/- are shown in the lighter font, and comparisons between +/- and -/- animals are in bold-fonts. Increases with respect to control are in red font and arrows (↑), and decreases are in blue font and arrows (↓). Only those changes found to be significant by ANOVA ($p < 0.01$) are presented, the missing values (-) were not significantly affected. Please see supplemental tables for results of individual tissues for unirradiated (see FIGURE 11) and X-irradiated (see FIGURE 12) animals, and results of one- two-and three-way ANOVA for significant interactions between gender, genotype and irradiation (See FIGURE 13).

[0078] GSH, the chief soluble cellular thiol and chemical antioxidant, was increased overall, in contrast to the GSH-linked antioxidant enzymes, which were generally

decreased. These findings suggest that RIP1 may function, perhaps through regulation or Rho/Rac pathways, in up-regulation of these enzymes. Thus, increase in ambient LOOH could be explained as a secondary effect of the loss of RIP1 due to decreased activities of GST, GPX, GR and G6PD, which normally metabolize LOOH and consume GSH.

5   Increased GSH levels observed would thus be secondary to decreased consumption of GSH rather than increased synthesis, since the rate limiting enzyme for GSH-synthesis, γ-GCS, was unchanged or decreased. Analyses of these parameters by individual tissues supported this assertion (FIGURE 11). The only tissue in which GSH, LOOH and TBARS were decreased was liver, where GST and GPX were increased. Changes in

10   oxidative stress parameters and antioxidant enzymes were generally concordant for most tissues for any given parameter, and the degree of change was generally greater in the RIP1 -/- animals as compared with the RIP1 +/- animals. Taken together, these findings confirm our hypothesis that loss of RALBP1 results in global increase in tissue oxidative stress and changes in levels of GSH-linked antioxidant enzymes.

15   [0079]   X-irradiation resulted in increase tissue oxidative stress with generally increased LOOH and TBARS in most tissues, and a greater degree of increase in RIP1 -/- as compared with the RIP1 +/- animals (FIGURE 12). TBARS levels were, however, actually somewhat decreased in liver. With few exceptions, radiation caused a further decrease in expression of the GSH-linked enzymes. These findings are likely a combined

20   effect of gender, genotype and irradiation which may affect the overall levels of these enzymes by causing varying levels of tissue damage (see results of ANOVA for one-way, two-way, and 3-way interactions in FIGURE 13).

[0080]   Whole mouse genome gene expression array was used to compare the effect of RIP1 knockout in heart tissue, an organ particularly severely affected in RIP1 -/- animals.

25   The microarray data was analyzed using commercially available software. The entire array of 34,560 genes was then filtered based on the criteria for stepwise up-regulation, which stated that there must be at least a 2 fold up-regulation on a given gene in the RIP1 -/- mouse as compared with the RIP1 +/+ mouse, and that the fold up-regulation between RIP1 +/+ and RIP1 +/- mouse multiplied by the fold up-regulation between the RIP1 +/-

30   and RIP1 -/- mouse should be within 20% of that observed between RIP1 +/+ and RIP1 -/- mouse. This criteria was chosen on the basis of results with GSH-linked enzymes in which step-wise up or down-regulation of each enzyme between RIP1 +/+ and RIP1 +/-

mouse multiplied by that between the RIP1 +/- and RIP1 -/- mouse was roughly equal to the change between RIP1 +/+ and RIP1 -/- mouse. Of the 7 genes which satisfied these criteria (TABLE 4), four were stress-induced or heat-shock induced proteins.

[0081]   For TABLE 4, a murine genome array was used to compare RIP1 +/+ vs. RIP1 +/-, RIP1 +/+ vs. RIP1 -/-,and RIP1 +/- vs. RIP1 -/-, each in duplicate and analyzed using IOBION software. Significant effects were selected by stipulating ≥ 2 fold increase, and by stipulating stepwise effects defined such that the up-regulation fold between RIP1 +/+ vs. RIP1 -/- is within 20% of the product of the up-regulation folds of RIP1 +/+ vs. RIP1 +/- and RIP1 +/- vs. RIP1 -/-. The 7 up-regulated genes satisfying these criteria are presented.

[0082]   **TABLE 4. Genes up-regulated in heart tissue of RIP1 knockout**

Description	(+/+) vs. (+/-)	(+/-) vs. (-/-)	(+/+) vs. (-/-)
heat shock protein	1.09	1.53	2
heat shock protein 1, alpha	1.38	1.36	2.19
heat shock protein Hsp40	1.08	2.09	2.27
105-kDa heat shock protein	1.12	2.57	2.35
25-kDa mammalian stress protein 1	1.41	1.54	2.21
Stress-induced phosphoprotein 1	1.56	1.37	2.08
insulin-like growth factor binding protein 5	2	1.72	3.62

[0083]   Heat Shock (Stress) Proteins [Hsp] are a family of proteins that vary in size
(10 kDa to 110 kDa) and perform two essential functions within the cell. At homeostasis
Hsp can behave as 'chaperones' assisting proper folding of and proper
compartmentalization of other proteins. Hsp can unfold and refold improperly folded
proteins into the proper orientation or assist in targeting them for degradation. In a stress
induced environment (temperature, xenobiotics, radiation, viral, and oxidative injury)
where a higher likely hood of denatured proteins can exist, Hsp can mediate by either re-
naturing the protein, degrading the protein, protecting the protein from becoming
denatured, or transporting it to a compartment where it can be degraded. All of these
actions assist the cell in maintaining its integrity. It is known that many Hsp are regulated
by Heat Shock Factor 1 (Hsf-1). Hsf-1 is a transcription factor that forms a ternary
complex with some of the Hsp (inactive form). Upon stress, the Hsp is released and Hsf-1
is allowed to bind to DNA, which up-regulates and increases the Hsp production assisting
in relief from the impending stress. It was recently discovered that Hsf1 forms a complex
with Ral Binding Protein 1. Upon stress, the Ral Signaling Pathway is activated and
RalBP1 is removed from the complex, which allows Hsf-1 to translocate into the nucleus
where it up-regulated the production of stress proteins. Thus, RLIP76 binding to Hsf-1
serves to inhibit Hsf-1 from increasing heat-shock protein RNA transcription. Our results
are consistent with this postulate since loss of RIP1 caused a stepwise up-regulation of
heat shock proteins.

[0084]   The present invention demonstrates stress-resistance mechanisms and the role
of GS-E transport in these mechanisms. The stress-defense functions of RLIP76 have

been strongly implicated in cell culture studies which show that it is induced within

minutes of exposure to a variety of stressors including radiant energy and oxidants, and

serves to decrease intracellular accumulation of GS-E. The formation of toxic and pro-

apoptotic α,β-unsaturated aldehydes is an obligate result of membrane lipid peroxidation

5   which is known to occur in response to radiant and oxidative stress. GSTs catalyze the

reversible conjugation of these aldehydes with GSH, and the resulting GS-E is potent

inhibitors of GSTs as well as GR. Thus, the removal of these conjugates through further

metabolism to mercapturic acids or transport from cells is critical, not only to prevent

inhibition of these important GSH-linked oxidant defense enzymes, but also to prevent

10   accumulation of the parent aldehydes that can arise from the reverse reaction favored by

accumulation of these GS-E.

[0085]   As such, RLIP76 serves a critical function in regulating cellular levels of these

α,β-unsaturated aldehydes which are known not only to be capable of cross-linking and

denaturing proteins through formation of Schiff's bases and alkylation but also to be

15   capable of triggering apoptosis once critical concentrations are reached. Induction of

heat-shock proteins as a defense in the absence of RLIP76 is entirely consistent with the

protein-denaturing effects of α,β-unsaturated aldehydes. Since oxidative stress which

results from hydroxyl-radical formation and formation of down-stream products of

oxidation are accepted as chemical mechanisms for the toxic effects of radiant as well as

20   chemical injuries, the function of RLIP76 in regulation of intracellular levels of these

end-products of oxidation is entirely consistent with the proposed role of RLIP76 as a

prominent radiation-defense.

[0086]   The linkage of RLIP76 to the Ral and Ras pathways and in particular to the

Rho/Rac pathway, which is known to control stress responses, is also of fundamental

25   significance and similar links have not been found for other transporters. Although clear

evidence has been provided for the interaction of RLIP76 with these pathways,

mechanistic explanations regarding how RLIP76 is involved in mediating a diverse array

of functions has previously been far from clear. Through its protein-protein binding

motifs in the C-terminal domain, it has clearly been shown to bind important signaling

30   proteins including the AP2 clathrin adaptor protein, POB1, CDK1, and Hsp90 as well as

Hsf1. Therefore, these proteins may be regulating some effector function of RLIP76. In

addition, RLIP76 may be functioning as a regulator of these signaling proteins.

[0087]   As described herein, RLIP76 has an effector function as an active nucleotidase which is capable of coupling ATPase activity with trans-membrane movement of several allocrites. [See also, S. S. Singhal et al, *Int J Oncol.* **22**, 365 (2003); S. Awasthi et al., *Biochemistry* **39**, 9327 (2000); S. Awasthi et al., *Biochemistry* **40**, 4159, (2001); S. Awasthi et al., *Int. J. Oncol.* **22**, 713 (2003); S. Awasthi et al., *Int. J. Oncol.* **22**, 721 (2003); S. Awasthi et al., *J. Clin. Invest.* **93**, 958 (1994); all citations herein incorporated by reference.] RLIP76 has a C-terminal domain of RLIP76 and is found both in membrane as well as cytosol, it contains an active ATPase domain. The present invention demonstrates that RLIP76 is a modular protein containing multiple domains which may perform distinct functions at distinct intracellular sites.

[0088]   The dramatic effect of RLIP76 liposomes in providing complete protection from radiation toxicity has direct implications for treatment of radiation toxicity. The very real risks of radiation poisoning as a result of a nuclear accident, nuclear bombs, or even terrorist attacks with "dirty-bombs," mandate the critical need for post-exposure treatment of radiation victims. As described herein, RLIP76 liposomes are excellent candidates for development as a radiation protective agent which may have broad applicability, particularly given that these liposomes are capable of delivering sustained levels of RLIP76 in all tissue, even brain. These findings also indicate that these liposomes may be useful as vehicles for delivery of drugs, antisense therapies and other therapies to the brain.

[0089]   Thus, RLIP76 displays distinct transport properties as a nonselective transporter of neutral and charged compounds, is involved in multidrug resistance, and plays a role in modulating cellular signaling that affects cell proliferation and cell death. As a proteoliposome, RLIP76 may be provided to a mammal to protect against xenobiotic toxicity. Similarly, transfection of cells with an effective portion of RLIP76 that enables transporter activity will promote xenobiotic protection, including protection from environmental or other chemicals (e.g., stress-induced, drug delivered, physiologically induced). Protection includes the treatment, inhibition, reduction, or prevention of accumulation in one or more cells of any chemical, that, when degraded, has the potential to damage these cells. This protection may be for environmental purposes, chemical procedures, or for mammals in need thereof.

[0090]   The present invention is also a method of reducing the effects of ionizing radiation on one or more cells in an organism comprising the step of contacting the organism with a liposome further comprising RLIP76 or an effective portion of RLIP76.

[0091]   Still another form of the present invention is a method of enhancing the export

5   of toxic compounds from mammalian cells comprising the step of contacting one or more mammalian cells with a liposome further comprising RLIP76 or an effective portion of RLIP76.

[0092]   The present invention is also a method of transfecting mammalian cells to enhance the transport of toxic compounds comprising the step of contacting the organism

10   with a liposome further comprising RLIP76 or an effective portion of RLIP76.

[0093]   Another form of the present invention is a method of transfecting mammalian cells to enhance the resistance to ionizing radiation comprising the step of contacting one or more mammalian cells with a liposome further comprising RLIP76 or an effective portion of RLIP76.

15   [0094]   In still another form, the present invention is a method of enriching mammalian cells to enhance their resistance to toxic compounds (including ionizing radiation) comprising the following step of contacting the organism with a liposome further comprising RLIP76 or an effective portion of RLIP76.

[0095]   In addition, the present invention is a proteoliposomal composition for the

20   treatment of toxic compound exposure comprising a liposome further comprising RLIP76 or an effective portion of RLIP76 and a chemotherapeutic agent. Another form of the present invention is a proteoliposomal composition for the treatment of toxic compound exposure comprising a liposome further comprising RLIP76 or an effective portion of RLIP76 and an effective dose of radiation therapy.

25   [0096]   In yet another form, the present invention is a protein composition that protects one or more cells against the harmful accumulation of toxic compounds comprising RLIP76 or an effective portion of RLIP76 and a ligand to RLIP76 that enhances transport activity of RLIP76.

[0097]   The present invention also embodies a kit for protecting one or more cells in an organism from the accumulation of one or more toxic compounds comprising an effective dose of a liposome further comprising RLIP76 or an effective portion of RLIP76 and an instructional pamphlet.

5   [0098]   The present invention also includes a method of enhancing the resistance of one or more mammalian cells to toxic compounds comprising the step of contacting one or more mammalian cells with a liposome further comprising RLIP76 or an effective portion of RLIP76.

[0099]   While specific alternatives to steps of the invention have been described

10   herein, additional alternatives not specifically disclosed but known in the art are intended to fall within the scope of the invention. Thus, it is understood that other applications of the present invention will be apparent to those skilled in the art upon reading the described embodiment and after consideration of the appended claims and drawing.